Research Article

Two episodes of remote ischemia preconditioning improve motor and sensory function of hind limbs after spinal cord ischemic injury

Salah Omar Bashir[1], Mohamed Darwesh Morsy[1,2], Dalia Fathy El Agamy[2]

[1]Department of Physiology, College of Medicine, King Khalid University, Abha, Saudi Arabia, [2]Department of Physiology, College of Medicine, Menoufia University, Shebeen Alkoom, Egypt

Objectives: To investigate the effect of one and two remote ischemia preconditioning episodes (1-RIPC or 2-RIPC, respectively) on neuro-protection after spinal cord ischemic injury (SCI) in rats.
Design: Experimental animal study.
Setting: College of Medicine, King Khalid University, Abha, KSA.
Interventions: Male rats (n = 10/group) were divided into control, sham, SCIRI, 1-RIPC + SCIRI, and 2-RIPC + SCIRI. SCI was induced by aortic ligation for 45 min and each RIPC episode was induced by 3 cycles of 10 min ischemia/10 min perfusion. The two preconditioning procedures were separated by 24 h.
Outcome measures: after 48 h of RIPC procedure, Tarlov's test, withdrawal from the painful stimulus and placing/stepping reflex (SPR) were used to evaluate the hind limbs neurological function. SC homogenates were used to measure various biochemical parameters.
Results: Motor and sensory function of hind limbs were significantly improved and levels of MDA, AOPPs, PGE2, TNF-α, and IL-6, as well as the activity of SOD, was significantly decreased in SC tissue in either 1 or 2 episodes of RIPC intervention. Concomitantly, levels of total nitrate/nitrite and eNOS activity were significantly increased in both groups. Interestingly, except for activity of SOD, eNOS and levels of nitrate/nitrite, the improvements in all neurological biochemical endpoint were more profound in 2-RIPC + SCIRI compared with 1-RIPC + SCIRI.
Conclusion: applying two preconditioning episodes of 3 cycles of 10 min ischemia/10 min perfusion, separated by 24 h, boost the neuro-protection effect of RIPC maneuver in rats after ischemic induced SCI in rats.

Keywords: Spinal cord, Remote ischemic preconditioning, Delayed, Protection, Aorta

Introduction

The incidence of paraplegia due to spinal cord injury (SCI) in patients undergo thoraco-abdominal aortic repair is 5–16%.[1–4] Similar findings have been also obtained in animal studies.[5,6] SCI is very common during the aortic peri-operative surgery where multiple mechanisms are involved.[7–12] Ischemic preconditioning (IPC), defined as a repetitive non-injurious ischemic episode and reperfusion that precede a longer ischemic insult, has been listed as the best endogenous cellular protective mechanism yielding the tissue more resistant to a subsequent IRI or hypoxic insult.[13] It can be performed experimentally in human and animal as a direct IPC (DIPC) or a remote IPC (RIPC), both of which have shown promising findings with the varied outcome against SCI.[1,6,14–16]

IPC protection on most tissues has two windows of protection within the nervous system: an early (acute) or late (delayed) preconditioning. The acute protective effect of IPC become apparent within minutes after the initial insult and dissipates over several hours.[17–19] This phase has been shown to be a protein-independent phase that is mediated by the release of low levels of ROS, bradykinin and adenosine, as well as activation of potassium adenosine triphosphate channels.[6,17,20–22] However, the protection reappears again 24–72 h later

Correspondence to: Mohamed Darwesh Morsy, Department of Physiology, College of Medicine, King Khalid University, Abha 61421, Saudi Arabia; Mobile Number: +966544495223; Fax: +966+966172251690; E-mail: morsydarwesh@yahoo.com

DOI 10.1080/10790268.2019.1600829

(delayed) post initial preconditioning and is mediated by the upregulation of antioxidant enzymes, heat shock proteins and inducible nitric oxide synthase (iNOS), which ultimately increases the release of NO[17,19,23–25] rendering the pretreated organ resistant to IRI for several days after the initial ischemic insult.[17–19] Activation of humeral and/or neural pathway has been shown to be involved in both phases of protection.[2,15]

Ripc has become the most clinically related and experimental used techniques to protection the heart, the brain, and the kidney after a hypoxic insult.[26–30] although different protocols with varied iPc cycles, occlusion time, and intervals between the iPc intervention and the ischemia induction were used, the effectiveness of rIpC has been also shown against the acute phase of sCi after aorta repair or occlusion in animal models as well as in human[12,15,27,31–34]

In addition, RIPC can induce spinal cord ischemia tolerance during late phase post aorta occlusion (delayed protection). In this regard, an early RIPC in the hind limbs of rabbits improved their neurological function after a single 12.5-minutes of preconditioning followed either 24 or 48 h later by 30-minutes of aortic occlusion improves the neurological function of the infarcted spinal cord.[32] In a similar model of rats, a reduced incidence of paraplegia and improvements in the neurological scores were reported with either 2 or 5 min of IPC 48 h before a 10-minutes aortic balloon occlusion[33], or after 3 cycles of 3-minutes ischemia/3 min reperfusion, 8 h before the occlusion of the abdominal aorta.[34] Importantly, as the duration of the intervening reperfusion period is increased, the neuro-protection of the IPC after a first preconditioning stimulus declines gradually and is eventually lost after 1–2 h. However, the provision of second preconditioning within the acute preconditioning stimulus reinstates the protective effects.[17,35] The authors concluded that additional preconditioning to the first one is always effective during the acute preconditioning phase, the longer the interval from the first preconditioning and the second, the more potent is the protective effect. Unfortunately, this has been only confirmed during the acute phase neuro-protection of RIPC.

So, the aim of the present study was to investigate the ability of the 2nd preconditioning episode to synergize and magnify the effectiveness of the first preconditioning episode in a rat model of ischemic reperfusion injury of the spinal cord (Table 1).

Material and methods

Animals

Adult male Sprague–Dawley rats were obtained from the Medical College at King Khalid University, Abha, KSA. All animals were housed under controlled temperature (23 ± 1°C), humidity (40−65%) and 12 h light/dark cycle. Animals were fed a normal diet and had free access to drinking water *ad libitum*. Ethics of this study were approved by the ethical committee of the College of Medicine at King Khalid University, Abha, Saudi Arabia which is in accordance to the animal use guidelines established by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

Experimental procedure

Forty rats were allocated five groups ($n = 10$) as follows into (1) a control group: remained with no surgical procedures or RIPC, (2) a sham-operated group: subjected to peritoneal cavity accessed with no aorta clamping or RIPC induction. (3) a spinal cord ischemic reperfusion injury-induced group (SC-IRI): underwent a surgical procedure to complete clamping of the aorta by microvascular clamp forceps for 45 min, as explained later without any preconditioning interventions. (4) a single exposure to RIPC (1X-RIPC + SCIRI): exposed to a single episode of RIPC 24 h before being exposed to aorta clamp occlusion (as mentioned Above) for a period of 45 min. (5) double exposure to RIPC (2-RIPC + SCIRI): exposed to two RIPC episodes every 24 h and then exposed to an aorta clamp (as mentioned above) for a period of 45 min. The experimental procedure is described in Figure 1. The aortic occlusion period was selected as paraplegia develops in rodents and larger animals, if the duration of aortic occlusion equals or exceeds 30 min.[34,37] The RIPC episode was selected as single episode of RIPC-induced by 3 cycles of 10 min ischemia/10 min reperfusion affords early protection against SCIRI.[34] Neurological function and tissue collections were done 48 h post the aortic clamp in all groups of rats.

Induction of SC-IRI

The procedure of anesthesia and induction of SC-IRI in rats of this study was done as previously described in our labs.[36] Rats were anesthetized with intraperitoneal (i.p.) injection of 60 mg/kg ketamine and 5 mg/kg xylazine and were given an intravenous (i.v.) dose of 150 IU/kg heparin. Rats were placed in a supine position of a heating pad to maintain body temperature at 37°C and their femoral and carotid arteries were cannulated with catheters to measure distal arterial pressure (DAP) and proximal arterial pressure (PAP), respectively. A midline laparotomy was conducted and a microvascular surgical clamp was placed over the abdominal aorta below the left renal vein and just above aortic

Table 1 Sensory assessment and placing stepping reflex in all groups of rats.

	Test	Control	Sham	SC-IRI	1XRIPC + SC-IRI	2XRIPC + SC-IRI	*P* value
Sensory assessment	Normal	10 (100%)	10 (100%)	2^{ab} (29%)	6^{abc} (67%)	8^{abcd} (89%)	$c^2 = P < .025$
	Affected	0 (0%)	0 (0%)	5^{ab} (71%)	3^{abc} (33%)	1^{abcd} (11%)	
Placing/stepping reflex	Normal	10 (100%)	10 (100%)	1^{ab} (14%)	4^{abc} (44%)	5^{abcd} (56%)	$c^2 = P < .025$
	Impaired	0 (0%)	0 (0%)	2^{ab} (29%)	5^{abc} (56%)	4^{abcd} (44%)	
	Lost	0 (0%)	0 (0%)	4^{ab} (57%)	0^{abc} (0%)	0^{abc} (0%)	

Notes: Results are expressed as number of rats and the percentage of number. Significance was considered when *P* value was <.05. a: vs. control; b: vs. Sham-operated; c: vs. SC-IRI and d: vs. 1-RIPC + SCIRI spinal cord ischemic reperfusion injury-induced group;. IX-RIPC: one episode of remote ischemia reperfusion injury. 2X-RIPC: two episodes of remote ischemia reperfusion injury separated by 24 h.

bifurcation for continuous complete occlusion of the aorta for 45 min. This has been confirmed by the immediate sustained decreased in distal aortic pressure. During the occlusion procedure, the proximal aortic pressure was maintained approximately at 40 mmHg by blood withdrawal in a collecting circuit filled with heparinized saline (4 U/ml) which was always maintained at 37°C. At the end of the ischemic period, reperfusion was done by releasing the aortic clamp and the heparinized blood was reperfused back to the rats over a period of 1 min.

The death rate in the control and sham group were 0% (zero rats), while in the SC-IRI group of rats is 30% (three rats). On the other hand, the death rate in 1XRIPC+SC-IRI and 2XRIPC+SC-IRI groups was 10% (one rat).

Neurological assessment

The motor function was assessed in each rats using Tarlov's Scoring which is well established in our labs.[36] In this test, each rat is given a score between 0.0 and –5, based on the following criteria (1) score 0 = no voluntary hind limb movement, (2) score 1 = movement of joints perceptible, (3) score 2 = active movement but unable to sit without assistance, (4) score 3 = able to sit but unable to hop, (5) score 4 = weak hop and (6) score 5 = complete recovery of hind limb function. Similarly, sensory function of hind limbs was assessed by the withdrawal of one limb from a painful stimulus[36] where score 0 indicates no ability to respond to the noxious stimulus and score 1 indicates a positive withdrawal response. Dragging the dorsum of the hind paw along the edge of a surface procedure was used to assess the placing/stepping reflex (SPR) of each rat.[36] This normally evokes a coordinating lifting and placing response which was graded as follows: 0 = normal;1 = weak; and 2 = no stepping.[36]

Blood and spinal cord collection and measurements

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). One ml of blood was directly collected

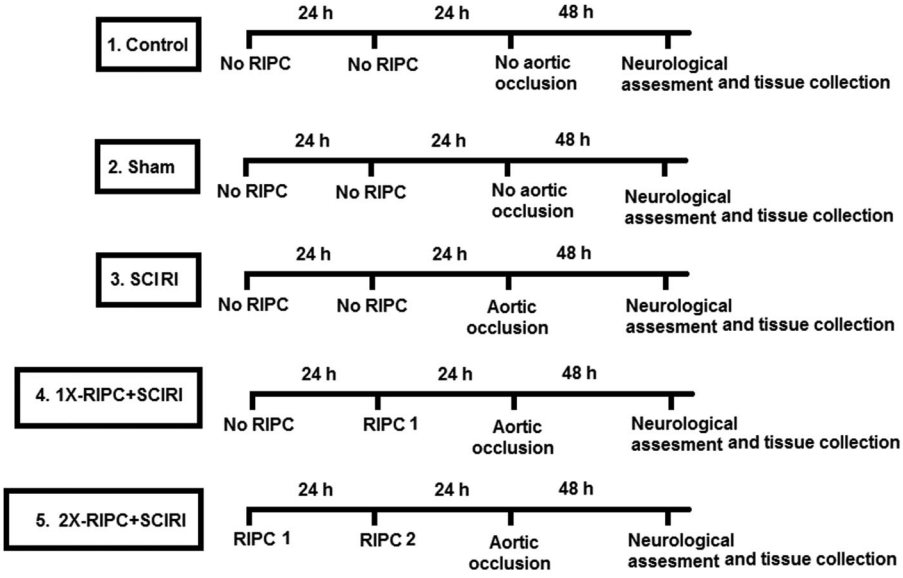

Figure 1 Experimental design of the study.

from each rat into EDTA tubes to collect plasma which was used later for measurements the levels of nitrite and nitrate using an assay kit (Cat. No. 780001, Cayman, Ann Arbor, MI, USA). Then, rats were sacrificed with a high dose of sodium thiopental intravenously for humanitarian propose and rapidly the spinal cord samples were collected from the 3rd, 4th and 5th lumbar segments. These tissue samples were collected and homogenized in ice-cold lysis buffer (pH 7.4) containing (0.1 M phosphate, 1 mM EDTA, and 10 μM indomethacin). To each sample, acetone was added to each homogenate (2:1 v:v). After that, all homogenates were centrifuged for 10 min. at 1500 rpm at 4°C to collect the supernatants which were stored at −80°C and used later to measure levels of Malondialdehyde (MDA) (Cat. No. NWK-MDA01, NWLSS, USA), advanced oxidation protein products (AOPP) (Cat. No. STA-318, Cell Biolabs, CA, USA) and prostaglandin E2 (PGE_2) (Cat. No. MBS262150, MyBioSource, CA, USA) as well as the activity of superoxide dismutase (SOD) (Cat. No. 706002, Cayman Chemical, Ann Arbor, MI, USA) and iNOS (Cat. No: MBS023874, Mybiosource, San Diego, USA).

Statistical analysis

All statistical analysis was done using GraphPad Prism statistical software package, version 6 (GraphPad Prism, San Diego, CA). All data were analyzed using one-way analysis of variance (ANOVA) followed by post-hoc Scheffe test. Data are expressed as means ± SD and a value of P values < .05 were considered statistically significant.

Results

Motor, SPR and sensory assessment

Motor function was evaluated using Tarlov's score test 48 h post SCI in all rat's. Figure 2 showed that the mean value of Tarlovs score (motor function) in SC-IRI group significantly decreased compares with both the control and the sham rat groups. Rats of 2XRIPC + SC-IRI showed significant elevation of their Tarlov's score when compared with SC-IRI group. On the other hand, the 1XRIPC group showed significant elevation of their Tarlov's score compared with both the control and sham groups but, insignificantly changed when compared with either SC-IRI or 2XRIPC groups. The sensory assessment test in the SC-IRI group showed a significant deterioration of the sensory function (71%) when compared with either control or sham group (0%). 1XRIPC + SC-IRI and 2XRIPC + SC-IRI groups appeared significant improvement in their sensory function (33% and 11%, respectively) when compared with SC-IRI rats (71%). 2XRIPC + SC-IRI group showed significant improvement in their sensory function (89%) when compared with 1XRIPC + SC-IRI group (67%). Regarding the placing/stepping reflex, the SC-IRI group showed significant impairment and lost (86% for both) when compared with either control or sham group (0%). Exposure to RIPC in both 1XRIPC + SC-IRI and 2XRIPC + SC-IRI groups resulted in significant improvement of their placing/stepping reflex test (44% and 56% respectively) when compared with SC-IRI group of rats. The 2XRIPC + SC-IRI group showed significant improvement of placing/stepping reflex test (56%) when compared with the 2XRIPC + SC-IRI group (44%).

Levels of inflammatory markers

There was no significant alteration in the SC levels of TNF-α or IL-6 in the control group when compared with the sham-operated rats. Significant elevations in the levels of both TNF-α and IL-6 were seen in the SC homogenates of SCIRI-induced rats (Figure 3A, B). However, both 1-RIPC and 2-RIPC groups have significantly lower levels of TNF-α and IL-6 in the SC homogenates of rats, compared with their corresponding levels measured in SCIRI-induced rats (Figure 3A, B). On the hand, 2-RIPC + SCIRI group has significantly lowered the levels of both cytokines in the SC homogenates compared with 1-RIPC + SCIRI, but there weren't any significant changes in their levels when 2-RIPC + SCIRI group was compared with either control or sham-operated rats (Figure 3A, B).

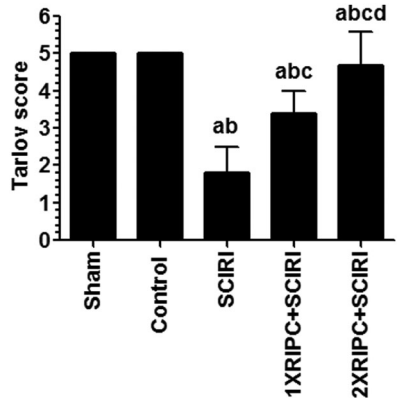

Figure 2 Tarlov scores in all groups of rats. Results are expressed as means ± SD (n = 10). Significance was considered when P value was <.05. a: vs. Control; b: vs. Sham-operated; c: vs. SC-IRI; d: vs. 1-RIPC + SCIRI;. SC-IRI: spinal cord ischemic reperfusion injury-induced group; IX-RIPC: one episode of remote ischemia reperfusion injury. 2X-RIPC: two episodes of remote ischemia reperfusion injury separated by 24 h.

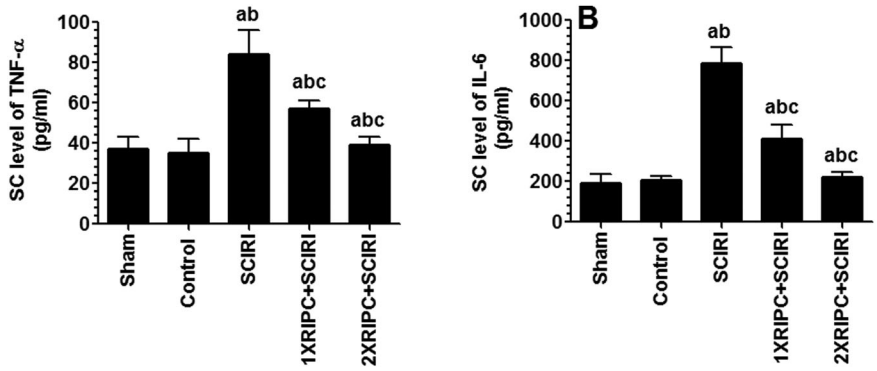

Figure 3 Spinal cord levels of TNF-α (A) and IL-6 (B) in all groups of rats. Results are expressed as means ± SD ($n = 10$). Significance was considered when P value was <.05. a: vs. Control; b: vs. Sham-operated; c: vs. SC-IRI; d: vs. 1-RIPC + SCIRI; SC-IRI: spinal cord ischemic reperfusion injury-induced group; IX-RIPC: one episode of remote ischemia reperfusion injury; 2X-RIPC: two episodes of remote ischemia reperfusion injury separated by 24 h.

Oxidative stress parameters and PGE2 levels in SC homogenates

No significant differences in the levels of MDA, AOPP, PGE_2 or in the activity of SOD were seen between control and sham-operated rats (Figure 4A–D). The levels of MDA, AOPP and PGE_2 and activity of SOD showed a significant elevation in the SC homogenates in SCIRI-induced rats compared with the control group (Figure 4A–D). While the levels of all these oxidative parameters decreased significantly in the SC homogenates in both 1-RIPC-induced and 2- RIPC-induced rats when compared to SCIRI group, with a more significant reduction in levels of MDA, AOPP, and PGE_2, but not SOD activity. Although the levels of MDA, AOPPS, and PGE_2 returned almost to the normal control levels in the SC homogenates of 2-RIPC + SCIRI-induced rats (Figure 4A, B, D), the activity of SOD remained significantly higher when compared with the control or the sham-operated rats and insignificantly changed when compared with 1-RIPC + SCIRI-induced rats (Figure 4C).

SC levels of total nitrate/nitrite and activity of eNOS

Similarly, levels of total nitrate/nitrite and activity of eNOS were not significantly altered between control and sham-operated rats (Figure 5A, B). However, significant increases in SC levels of total nitrate/nitrite and eNOS activity were seen in SCIRI-induced rats as compared to control or sham-operated rats. On the other hand, levels of total nitrate/nitrite were significantly increased more in the SC homogenates collected from both SCIRI-induced rats received either 1 or 2-RIPC intervention as compared to all other groups of the study (Figure 5A, B). However, No significant differences were detected in the SC levels of total nitrate/nitrite and activity of eNOS when 1-RIPC + SCIRI and 2-RIPC + SCIRI were compared to each other (Figure 5A, B).

Discussion

RIPC acts within two phases of protection, an acute phase which lasts for 1–2 h and a delayed phase that may acts after 24 h up to several days.[17] Interestingly but during the acute phase of protection, it has been suggested that the addition of a second preconditioning stimulus could exaggerate the protective effect of the first stimulus during the delayed phase by boosting the protective transcriptional events that occur during this phase.[17,35] In addition, the effect of the second stimulus becomes more potent as the time interval between the two stimuli is longer.[35] Hence, it was of our interest in this study to examine the effect of 2 preconditioning stimuli separated by 24 h on the neurological outcome measured 48 h post the aortic occlusion-induced SCI in rats.

The first observation of this study support the suggestion of Iliodromitis *et al.*[35] during acute RIPC and shows that the addition of the 2nd preconditioning stimulus 24 h after an initial preconditioning stimulus, produced a more profound improvement in sensory and motor function of the hind limbs as compared to those measured in rats received a single episode of I/R preconditioning. This was evident by the higher scores obtained from the neurological examination of the limb withdrawal from a painful stimulus, placing/stepping reflex (SPR) and Tarlov's scoring system, 48 h post the aortic occlusion. These results encouraged us to go further looking for possible mechanisms of action that be exaggerated by the application of a 2nd stimulus during this protocol of RIPC.

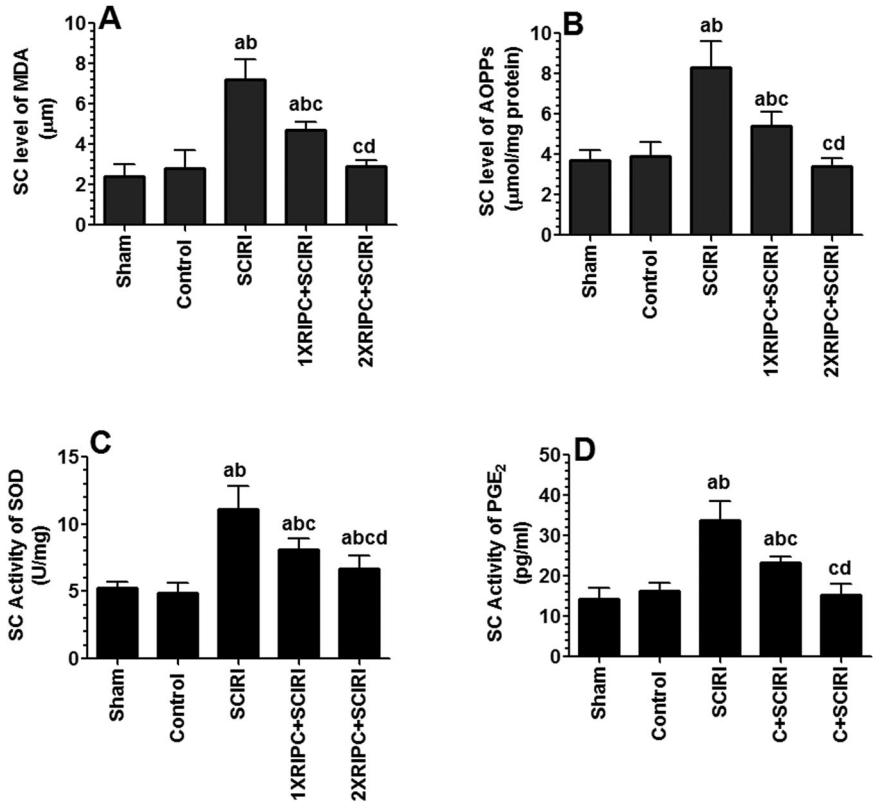

Figure 4 Spinal cord levels of Malondialdehyde (MDA, A), advanced oxidation protein products (AOPP, B), and PGE_2 (D) and activity of superoxide dismutase (SOD, C) in all groups of rats. Results are expressed as means ± SD ($n = 10$). Significance was considered when P value was <.05. a: vs. Control; b: vs. Sham-operated; c: vs. SC-IRI; d: vs. 1X-RIPC + SCIRI;. SC-IRI: spinal cord ischemic reperfusion injury-induced group; IX-RIPC: one episode of remote ischemia reperfusion injury. 2X-RIPC: two episodes of remote ischemia reperfusion injury separated by 24 h.

The excessive amounts of ROS generated from multiple resources during the SCI as well as the active inflammation of infiltrating macrophages and neutrophils and activated microglia and astrocytes are the major effectors in the delayed insult after induction of SCIRI where their inhibition is one of the most important strategies in early treatment for SCI.[38–49] In confirmation, in the current study, levels of lipid peroxides and protein oxidative stress markers namely, MDA and AOPP as well as in the inflammatory markers including TNF-α, IL6 and PGE_2 were significantly raised in the SC homogenates, 48 h post

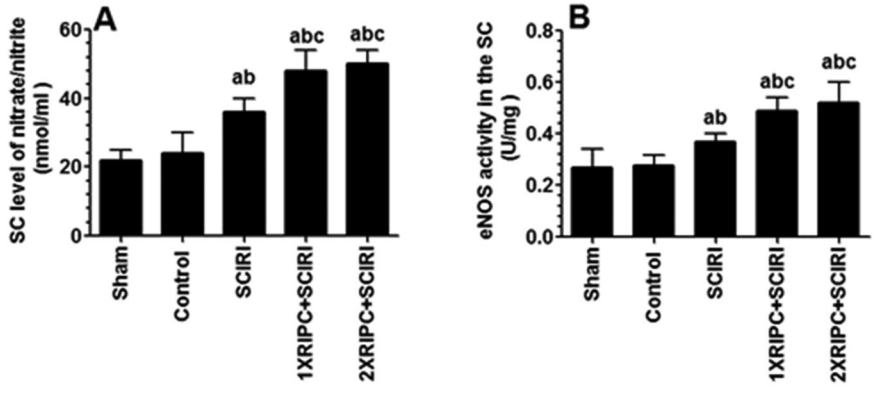

Figure 5 Spinal cord levels of nitrate/nitite (A) and activity of eNOS (B) in all groups of rats. Results are expressed as means ± SD ($n = 10$). Significance was considered when P value was <.05. a: vs. Control; b: vs. Sham-operated; c: vs. SC-IRI; d: vs. 1-RIPC + SCIRI; SC-IRI: spinal cord ischemic reperfusion injury-induced group;. IX-RIPC: one episode of remote ischemia reperfusion injury. 2X-RIPC: two episodes of remote ischemia reperfusion injury separated by 24 h.

SCIRI induction. In spinal cord studies, a single episode of RIPC was shown to reduce the levels of MDA indicating that free radical-mediated cellular damage was prevented.[50] In support, levels of all the above mentioned oxidative stress and inflammatory parameters were significantly reduced in the SC tissue exposed to SCIRI and received either single or double RIPC intervention with a more significant decrease to be associated with the application of two stimuli RIPC. The SC content of ROS and MDA are a determinant factor for motor and sensory functional recovery after SCI.[51,52] These data could explain why these rats had better motor and sensory functions recovery and suggest a magnification of the delayed protection response, which was our second objective of this study.

Previously, it was shown that low amounts of rOs might be sufficient to serve as part of the rIpC mechanism to modify cellular activities and prepare the cell against a more severe hypoxic insult.[53,54] during the delayed phase of protection of various types iPc, the consensus from various authors have shown that sOd, hSps, and enOs are the major protein which upregulated to afford the desired neuro-protection.[17] Notably, the delayed protective mechanism of rIpC is mediated via rOs-dependent pathway via an increased cytoprotective antioxidant response that is mediated mainly by upregulation of the antioxidant enzymes, sOd and MnSOD.[12,55,56]

Surprisingly and unexpectedly, we have found significant increases in the levels of SOD in the SC homogenates of SCIRI-induced rats where it was significantly decreased in RIPC treated rats, with a trend to more significant decreases when the two preconditioning episodes were applied and separated by 24 h. In fact, similar increases in SOD activity after a similar protocol to induce SCIRI has been also reported in our previous study.[36] This could be a response to overcome the increased ROS generated after the I/R injury. In addition, although it is becoming increasingly clear that induction of antioxidant enzymes may be an important mechanism involved in delayed preconditioning, the relative contribution of specific antioxidant enzymes is unclear. Other important antioxidant enzymes such as catalase could account many important roles during IPC. An example is supported by the study of Li *et al*.[57] where hearts isolated from transgenic mice in which myocardial catalase is increased 60-fold (while SOD and glutathione peroxidase were not altered), were resistant to I/R-induced injury. Unfortunately, we didn't measure the activities or expression of other antioxidant enzyme or their upstream regulator, Nrf2 to deeply explain our results which can be considered as a limitation of this study.

On the other hand, the role of TNF-α during IPC remains largely unclear and contradictory conclusions still exist. Although it is an inflammatory and cell death-promoting agent, TNF-α may mediate several beneficial effects during IPC by acting to suppress the production of other pro-inflammatory cytokines and/or enhance the expression and function of different transporters and enzymes.[58–60]

However, findings of our study support the pro-inflammatory role of TNF-α. Both protocols of RIPC used in this study resulted in significant decreases in TNF-α with parallel decreases in its targets, IL6 and PGE_2. Similar to these findings, levels of TNF-α were significantly reduced in RIPC-treated rats possibly due to preservation of antioxidants induced by IPC.[27] In spinal cord injury studies, RIPC attenuated recruitment of leukocytes into the inflammatory site.[50] Moreover, the interplay and complex formation between the neutrophils and platelets was attenuated by RIPC.[61] Furthermore, RIPC after brain IRI reduced the number of adherent leukocytes in cerebral vessels after hypothermic circulatory arrest.[62]

On the other hand, other studies have shown that the higher levels of NO generated during IRI within a tissue, the more protection is provided.[63] This has been confirmed in the heart, brain and skeletal muscles where the treatments with L-Name, a NOS inhibitor prevented the development of IPC delayed protection.[18,63–65] In fact, it was shown that NO has biphasic protection during both the acute and delayed phases of protection after IPC.[63,65] This has been explained by the presence of different isoforms of NOS in different phases, and the differential regulation of these isoforms.[63]

However, it seems that the upregulation of eNOS is most important during the late phase after RIPC in the brain after focal ischemia which is associated with the activation of extracellular signal-regulated protein kinase (ERK) and AKT Survival pathway.[65–67] It is noteworthy that neutral results of NO actions have been reported in RIPC spinal cord studies in rabbits where the authors induced RIPC using 5 min ischemia/15 min reperfusion followed by 49 min aorta occlusion.[50] In contrast, our results show the opposite and highlight the important role of eNOS and NO in the delayed protection of RICP after the SCIRI. In this study, levels of nitrate/nitrite and eNOS were significantly increased in SCIRI-induced rats. This could be a transient adaptive protective mechanism to ameliorate the damaging effect of ROS and inflammation. However, Further significant increases in the levels of

nitrate/nitrite and eNOS were significantly increased in the SC homogenates of the SCIRI-induced rats underwent the single and double preconditioning procedure with no significant difference between these two RIPC interventions, suggesting that although NO mediates RIPC delayed protective effect but it is not involved in the mechanism by which the second stimulus acts. Hence, it will worthy to investigate other mediators of the delayed protection such as the effect of these protocols of expression levels of HSPs in future trails.

Conclusion

The finding of our current study showed that the RIPC achieved by applying two equal episodes of RIPC (three cycles of 10 min ischemia/10 min reperfusion) has a more potent effect to reduce the neurological impairments in rats after IRI, as compared to the protective effect of a single episode of a similar RIPC. This is associated with more profound antioxidant and anti-inflammatory effect as well as trends to increase the activity of eNOS and bioavailability of NO.

Acknowledgements

First of all, the authors thank the deanship of scientific research, King Khalid University, KSA that funding and continuously support this work. The authors highly appreciate the extreme help of all staff members of Abha General Hospital. The authors also thank greatly, Ahmed M Darwesh, Dr. Khalid S Bashir, and Omar M Morsy for their help in the collection, processing of samples and performing the statistical analysis of data. We extend our thanks to all staff members and technicians of the Physiology and Biochemistry Departments of Colleges of Medicine, King Khalid University, Saudi Arabia, and College of Medicine, Menoufia University, Egypt; for their great help in this work.

Disclaimer Statements

Contributors None.

Funding Statement: This research was fully funded by a grant from the Deanship of Scientific Research King Khalid University, KSA, Project No. 511-2018G.

Conflicts of interest: All authors declare that they have no conflicts of interest.

Ethics approval None.

References

1 Toumpoulis IK, Anagnostopoulos CE, Drossos GE, Malamou-Mitsi VD, Pappa LS, Katritsis DG. Does ischemic preconditioning reduce spinal cord injury because of descending thoracic aortic occlusion? J Vasc Surg. 2003;37(2):426–32.
2 Coselli JS, LeMaire SA, Conklin LD, Koksoy C, Schmittling ZC. Morbidity and mortality after extent II thoracoabdominal aortic aneurysm repair. Ann Thorac Surg. 2002;73(4):1107–16.
3 Svensson LG, Crawford ES, Hess KR, Coselli JS, Safi HJ. Experience with 1509 patients undergoing thoracoabdominal aortic operations. J Vasc Surg. 1993;17(2):357–68.
4 Estrera AL, Miller CC III, Huynh TT, Porat E, Safi HJ. Neurologic outcome after thoracic and thoracoabdominal aortic aneurysm repair. Ann Thorac Surg. 2001;72:1225–30.
5 Isbir CS, Ak K, Kurtkaya O, Zeybek U, Akgün S, Scheitauer BW, *et al.* Ischemic preconditioning and nicotinamide in spinal cord protection in an experimental model of transient aortic occlusion. Eur J Cardiothorac Surg. 2003;23(6):1028–33.
6 Sapmaz A1, Ulus AT, Turan NN, Kaymaz FF, Yazıcıoğlu H, Ersöz S1, *et al.* Which type of conditioning method protects the spinal cord from the ischemia-reperfusion injury in 24 hs? Vascular. 2015;23(6):614–21.
7 Barone GW, Joob AW, Flanagan TL, Dunn CE, Kron IL. The effect of hyperemia on spinal cord function after temporary thoracic aortic occlusion. J Vasc Surg. 1988;8(4):535–40.
8 Ulus F, Hellberg A, Ulus AT, Karacagil S. Alterations in cerebrospinal fluid PO2, PCO2 and pH measurements during and after experimental thoracic aortic cross-clamping. Ann Vasc Surg. 2009;23(1):122–27.
9 Gharagozloo F, Neville Jr RF, Cox JL. Spinal cord protection during surgical procedures on the descending thoracic and thoracoabdominal aorta: a critical overview. Semin Thorac Cardiovasc Surg. 1998;10(1):73–86.
10 Neumar RW. Molecular mechanisms of ischemic neuronal injury. Ann Emerg Med. 2000;36(5):483–506.
11 Etz CD, Homann TM, Luehr M, Kari FA, Weisz DJ, Kleinman G, *et al.* Spinal cord blood flow and ischemic injury after experimental sacrifice of thoracic and abdominal segmental arteries. Eur J Cardiothorac Surg. 2008;33(6):1030–38.
12 Dong HL, Zhang Y, Su BX, Zhu ZH, Gu QH, Sang HF, *et al.* Limb remote ischemic preconditioning protects the spinal cord from ischemia–reperfusion injury. Anesthesiology. 2010;112(4):881–91.
13 Cohen MV, Downey JM. Is it time to translate ischemic preconditioning's mechanism of cardioprotection into clinical practice? J Cardiovasc Pharmacol Ther. 2011;16(3–4):273–80.
14 Fan T, Wang CC, Wang FM, Cheng F, Qiao H, Liu SL, *et al.* Experimental study of the protection of ischemic preconditioning to spinal cord ischemia. Surg Neurol. 1999;52(3):299–5.
15 Haapanen H, Herajärvi J, Arvola O, Anttila T, Starck T, Kallio M, *et al.* Remote ischemic preconditioning protects the spinal cord against ischemic insult: an experimental study in a porcine model. J Thorac Cardiovasc Surg. 2016;151(3):777–85.
16 Fukui T, Ishida K, Mizukami Y, Shimamoto K, Harada H, Yamashita A, *et al.* Remote ischemic preconditioning protects the spinal cord against ischemic insult: an experimental study in a porcine model. J Thorac Cardiovasc Surg. 2016;151(3):777–85.
17 Korthuis RJ, Gute DC, Cepinska G, Kvietys PR. Cellular mechanisms of acute versus delayed preconditioning. Pathophysiology. 1998;5(1):35–48.
18 Bolli R, Manchikalapudi S, Tang XL, Takano H, Qiu Y, Guo Y, *et al.* The protective effect of late preconditioning against myocardial stunning in conscious rabbits is mediated by nitric oxide synthase. Circ Res. 1997;81(6):1094–107.
19 Yu QJ, Wang YL, Zhou QS, Huang HB, Tian SF, Duan DM. Effect of repetitive ischemic preconditioning on spinal cord ischemia in a rabbit model. Life Sci. 2006;79(15):1479–83.
20 Tapuria N, Kumar Y, Habib MM, Abu Amara M, Seifalian AM, Davidson BR. Remote ischemic preconditioning: a novel protective method from ischemia reperfusion injury – a review. J Surg Res. 2008;150(2):304–30.
21 Ishida T, Yarimizu K, Gute DC, Korthuis RJ. Mechanisms of ischemic preconditioning. Shock. 1997;8(2):86–94.
22 Pagliaro P, Gattullo D, Rastaldo R, Losano G. Ischemic preconditioning. Life Sci. 2001;69(1):1–15.
23 Zhou X, Zhai X, Ashraf M. Direct evidence that initial oxidative stress triggered by preconditioning contributes to second window

of protection by endogenous antioxidant enzyme in myocytes. Circulation. 1996;93:1177–84.
24 Nayeem MA, Hess ML, Qian YZ, Loesser KE, Kukreja RC. Delayed preconditioning of cultured adult rat cardiac myocytes: role of 70 and 90 kDa heat stress proteins. Am J Physiol. 1997; 273(2 Pt 2):H861–68.
25 Imagawa J, Yellon DM, Baxter GF. Pharmacological evidence that inducible nitric oxide synthase is a mediator of delayed preconditioning. Br J Pharmacol. 1999;126(3):701–8.
26 Ali ZA, Callaghan CJ, Lim E, Ali AA, Nouraei SA, Akthar AM, *et al.* Remote ischemic preconditioning reduces myocardial and renal injury after elective abdominal aortic aneurysm repair: a randomized controlled trial. Circulation. 2007;116(11):98–105.
27 Hu S, Dong HL, Li YZ, Luo ZJ, Sun L, Yang QZ, *et al.* Effects of remote ischemic preconditioning on biochemical markers and neurologic outcomes in patients undergoing elective cervical decompression surgery. J Neurosurg Anesthesiol. 2010;22:46–52.
28 Jensen HA, Loukogeorgakis S, Yannopoulos F, Rimpiläinen E, Petzold A, Tuominen H, *et al.* Remote ischemic preconditioning protects the brain against injury after hypothermic circulatory arrest. Circulation. 2011;123(7):714.
29 Meybohm P, Bein B, Brosteanu O, Cremer J, Gruenewald M, Stoppe C, *et al.* A multicenter trial of remote ischemic preconditioning for heart surgery. N Engl J Med. 2015;373(15):1397–1407.
30 Hausenloy DJ, Candilio L, Evans R, Ariti C, Jenkins DP, Kolvekar S, *et al.* Remote ischemic preconditioning and outcomes of cardiac surgery. N Engl J Med. 2015;373(15):1408–17.
31 Herajärvi J, Anttila T, Sarja H, Mustonen C, Haapanen H, Mäkelä T, *et al.* Exploring spinal cord protection by remote ischemic preconditioning: an experimental study. Ann Thorac Surg. 2017;103(3):804–11.
32 Munyao N1, Kaste M, Lindsberg PJ. Tolerization against loss of neuronal function after ischemia–reperfusion injury. Neuroreport. 1998;9(2):321–5.
33 Abraham VS, Swain JA, Forgash AJ, Williams BL, Musulin MM. Ischemic preconditioning protects against paraplegia after transient aortic occlusion in the rat. Ann Thorac Surg. 2000; 69(2):475–9.
34 Selimoglu O, Ugurlucan M, Basaran M, Gungor F, Banach M, Cucu O, *et al.* Efficacy of remote ischaemic preconditioning for spinal cord protection against ischaemic injury: association with heat shock protein expression. Folia Neuropathol. 2008;46(3): 204–12.
35 Iliodromitis EK, Papadopoulos C, Paraskevaidis IA, Kyriakides ZS, Flessa C, Kremastinos DT. Protection from preconditioning can be reinstated at various reperfusion intervals. Cardiovasc Drugs Ther. 1996;10(3):341–6.
36 Morsy MD, Mostafa OA, Hassan WN. A potential protective effect of α-tocopherol on vascular complication in spinal cord reperfusion injury in rats. J Biomed Sci. 2010;17(55):55–64.
37 Parrino PE, Kron IL, Ross SD, Shockey KS, Fisher MJ, Jr GJ, *et al.* Retrograde venous perfusion with hypothermic saline and adenosine for protection of the ischemic spinal cord. J Vasc Surg. 2000;32:171–8.
38 Schulz R, Czaja SJ, Lustig A, Zdaniuk B, Martire LM, Perdomo D. Improving the quality of life of caregivers of persons with spinal cord injury: a randomized controlled trial. Rehabil Psychol. 2009;54(1):1–15.
39 Ellenbroek D, Kressler J, Cowan RE, Burns PA, Mendez AJ, Nash MS. Effects of prandial challenge on triglyceridemia, glycemia, and pro-inflammatory activity in persons with chronic paraplegia. J Spinal Cord Med. 2015;38(4):468–75.
40 Zhu P, Li J, Fujino M, Zhuang J, Li X. Development and treatments of inflammatory cells and cytokines in spinal cord ischemia-reperfusion injury. Mediators Inflamm. 2013;2013:1–7.
41 Yamanaka K, Sasaki N, Fujita Y, Kawamoto A, Hirata K, Okita Y. Impact of acquired and innate immunity on spinal cord ischemia and reperfusion injury. Gen Thorac Cardiovasc Surg. 2016;64(5): 251–9.
42 Hall ED, Yonkers PA, Horan KL, Braughler JM. Correlation between attenuation of posttraumatic spinal cord ischemia and preservation of tissue vitamin E by the 21-aminosteroid U74006F: evidence for an in vivo antioxidant mechanism. J Neurotrauma. 1989;6(3):169–76.
43 Cuzzocrea S, Riley DP, Caputi AP, Salvemini D. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. Pharmacol Rev. 2001;53(1): 135–59.
44 Waters S, Fae A, Gondalia J, Holm J, Karlström L, Nilsson U, *et al.* Effects of pretreatment with a xanthine oxidase inhibitor on free radical levels during carotid endarterectomy. Free Radic Res. 2004;38(3):283–93.
45 Stys PK. Anoxic and ischemic injury of myelinated axons in CNS white matter: from mechanistic concepts to therapeutics. J Cereb Blood Flow Metab. 1998;18(1):2–25.
46 Silva NA, Sousa N, Reis RL, Salgado AJ. From basics to clinical: a comprehensive review on spinal cord injury. Prog Neurobiol. 2014;114:25–57.
47 Banovac K, Williams JM, Patrick LD, Levi A. Prevention of heterotopic ossification after spinal cord injury with COX-2 selective inhibitor (rofecoxib). Spinal Cord. 2004;42(12):707–10.
48 Hillard VH, Peng H, Zhang Y, Das K, Murali R, Etlinger JD, *et al.* Tempol, a nitroxide antioxidant, improves locomotor and histological outcomes after spinal cord contusion in rats. J Neurotrauma. 2004;21(10):1405–14.
49 Wang CH, Zhu LL, Ju KF, Liu JL, Li KP. Anti-inflammatory effect of delphinidin on intramedullary spinal pressure in a spinal cord injury rat model. Exp Ther Med. 2017;14(6):5583–8.
50 Gurcun U, Discigil B, Boga M, Ozkisacik E, Badak MI, Yenisey C, *et al.* Is remote preconditioning as effective as direct ischemic preconditioning in preventing spinal cord ischemic injury? J Surg Res. 2006;135(2):385–93.
51 Wilson JR, Grossman RG, Frankowski RF, Kiss A, Davis AM, Kulkarni AV, *et al.* A clinical prediction model for long-term functional outcome after traumatic spinal cord injury based on acute clinical and imaging factors. J Neurotrauma. 2012;29(13): 2263–71.
52 Rosety-Rodriguez M, Camacho A, Rosety I, Fornieles G, Rosety MA, Diaz AJ, *et al.* Low-grade systemic inflammation and leptin levels were improved by arm cranking exercise in adults with chronic spinal cord injury. Arch Phys Med Rehabil. 2014;95(2): 297–302.
53 Ambrosio G, Tritto I, Chiariello M. The role of oxygen free radicals in preconditioning. J Mol Cell Cardiol. 1995;27(4):1035–39.
54 Thompson JW, Narayanan SV, Perez-Pinzon MA. Redox signaling pathways involved in neuronal ischemic preconditioning. Curr Neuropharmacol. 2012;10(4):354–69.
55 Hu S, Dong H, Zhang H, Wang S, Hou L, Chen S, *et al.* Noninvasive limb remote ischemic preconditioning contributes neuroprotective effects via activation of adenosine A1 receptor and redox status after transient focal cerebral ischemia in rats. Brain Res. 2012;1459:81–90.
56 Mehrjerdi F Z, Aboutaleb N, Habibey R, Ajami M, Soleimani M, Arabian M, *et al.* Increased phosphorylation of mTOR is involved in remote ischemic preconditioning of hippocampus in mice. Brain Res. 2013;1526:94–101.
57 Li G, Chen Y, Saari JT, Kang YJ. Catalase-overexpressing transgenic mouse heart is resistant to ischemia-reperfusion injury. Am j Physiol. 1997;273(3 Pt 2):H1090–5.
58 Meller R, Simon RP. A critical review of mechanisms regulating remote preconditioning-induced brain protection. J Appl Physiol. 2015;119(10):1135–42.
59 Konstantinov IE, Arab S, Kharbanda RK, Li J, Cheung MMH, Cherepanov V, *et al.* The remote ischemia preconditioning stimulus modifies inflammatory gene expression in humans. Physiol Genomics. 2004;19(1):143–50.
60 Pradillo JM, Hurtado O, Romera C, Cardenas A, Fernandez-Tome P, Alonso-Escolano D, *et al.* Tnfr1 mediates increased neuronal membrane EAAT3 expression after in vivo cerebral ischemic preconditioning. Neuroscience. 2006;138(4):1171–78.
61 Kharbanda RK, Mortensen UM, White PA, Kristiansen SB, Schmidt MR, Hoschtitzky JA, *et al.* Transient limb ischemia induces remote ischemic preconditioning in vivo. Circulation. 2002;106(23):2881–3.
62 Yannopoulos FS, Arvola O, Haapanen H, Herajärvi J, Miinalainen I, Jensen H, *et al.* Leg ischaemia before circulatory arrest alters brain leucocyte count and respiratory chain redox state. Interact Cardiovasc Thoracic Surg. 2014;18(3):272–7.

63 Zhao H, Sun X, Xian X, Li W, Zhang M, Li Q. The role of nitric oxide in the neuro-protection of limb ischemic preconditioning in rats. Neurochem Res. 2007;32(11):1919–26.
64 Pudupakkam S, Harris KA, Jamieson WG, DeRose G, Scott JA, Carson MW, *et al.* Ischemic tolerance in skeletal muscle: role of nitric oxide. Am J Physiol. 1998;275(1 Pt 2):H94–9.
65 Vlasov TD, Korzhevskii DE, Polyakova EA. Ischemic preconditioning of the rat brain as a method of endothelial protection from ischemic/repercussion injury. Neurosci Behav Physiol. 2005;35(6):567–72.
66 Peng B, Guo Q, He Z, Ye Z, Yuan Y, Wang N, *et al.* Remote ischemic postconditioning protects the brain from global cerebral ischemia/reperfusion injury by up-regulating endothelial nitric oxide synthase through the PI3K/Akt pathway. Brain Res. 2012; 1445:92–102.
67 Pignataro G, Esposito E, Sirabella R, Vinciguerra A, Cuomo O, Di Renzo G, *et al.* Nnos and p-ERK involvement in the neuro-protection exerted by remote postconditioning in rats subjected to transient middle cerebral artery occlusion. Neurobiol Dis. 2013;54: 105–14.